Analysis of Drug/Plasma Protein Interactions by Means of Asymmetrical Flow Field-Flow Fractionation

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Purpose. The applicability of Asymmetrical Flow Field-Flow Fractionation (Asymmetrical Flow FFF) as an alternative tool to examine the distribution of a lipophilic drug (N-Benzoyl-staurosporine) within human plasma protein fractions was investigated with respect to high separation speed and loss of material on surfaces due to adsorption.

Methods. Field-Flow Fractionation is defined as a group of pseudo-chromatographic separation methods, where compounds are separated under the influence of an externally applied force based on differences in their physicochemical properties. This method was used to separate human plasma in its protein fractions. The drug distribution in the fractions was investigated by monitoring the fractionated eluate for drug content by fluorescence spectroscopy.

Results. Human plasma was separated into human serum albumin (HSA), high density lipoprotein (HDL), α_2-macroglobulin and low density lipoprotein (LDL) fractions in less than ten minutes. Calibration of the system and identification of the individual fractions was performed using commercially available protein reference standards. The influence of membrane type and carrier solution composition on the absolute recovery of N-Benzoyl-staurosporine and fluorescein-isothiocyanate-albumin (FITC-albumin) was found to be quite significant. Both factors were optimized during the course of the investigations. N-Benzoyl-staurosporine was found to be enriched in the fraction containing HSA.

Conclusions. If experimental conditions are thoroughly selected and controlled to suppress drug and plasma protein adsorption at the separation membrane, Asymmetrical Flow FFF shows high recoveries and fast separation of human plasma proteins, and can be a reliable tool to characterize drug / plasma protein interactions. For analytical purposes it has the potential to rival established technologies like ultracentrifugation in terms of ease-of-use, precision, and separation time.

KEY WORDS: asymmetrical flow field-flow fractionation; FFF; plasma proteins; HAS; lipoproteins; N-Benzoyl-staurosporine; recovery; separation.

INTRODUCTION

Knowledge of interaction mechanisms between drugs and plasma proteins is of crucial importance for understanding the pharmacodynamics and -kinetics of a drug. Drug binding influences the distribution, excretion, metabolism and interaction with the drug receptors. Differences in protein binding of a drug among humans and across species may explain differences in drug action between patients and in different species (1,2).

Drugs in blood circulation are mainly transported by human serum albumin (HSA), α_1-acidic glycoprotein (α_1-AGP) and lipoproteins (high density lipoprotein, HDL; low density lipoprotein, LDL; very low density lipoprotein, VLDL). HSA is by far the most abundant plasma protein with a concentration of about 40 g/l. Most drugs—being acidic, neutral, or basic in nature—are bound to HSA (1). α_1-AGP is a globulin and shows large fluctuations in concentration due both to physiological and pathological conditions. It binds mainly basic drugs as well as some acidic drugs (3). Lipoproteins are known to be the carriers of cholesteryl esters and to bind significant amounts of lipophilic drugs (4). They have, however, received little attention as potential drug binding sites for drugs in plasma compared to HSA and α_1-acidic glycoprotein.

Standard methods to investigate the affinity and distribution of drugs to different plasma proteins are e.g. density gradient ultracentrifugation, electrophoresis and column liquid chromatography. These methods quite often are time consuming and/or require harsh and non physiological conditions during separation (5–7).

In difference to this Flow Field-Flow Fractionation (Flow FFF), a recently established analytical separation method, offers mild separation characteristics and is suitable for protein separation over a broad size range in short analysis time. The general feasibility of this method to separate plasma proteins has been demonstrated earlier (8–11), however, no recovery studies of the injected proteins have been performed. Also, no investigations on the influence of the specific properties of the membrane nor of the carrier liquid composition on the degree of adsorption of the diluted plasma protein fractions have been reported.

In this paper we report studies of drug/plasma protein interactions using asymmetrical Flow FFF as a separation method. N-Benzoyl-staurosporine (NBS) was used as a lipophilic model drug. In order to obtain quantitative information on interactions between model drug and plasma proteins, it is imperative that recovery studies of all components of interest are carried out, since selective adsorption of one of the components to the membrane could completely distort the picture.

MATERIALS AND METHODS

Materials

Lipoprotein standards were obtained from Calbiochem-Novabiochem, Juro Supply AG (Lucerne, Switzerland). FITC-

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ABBREVIATIONS AND SYMBOLS: D, diffusion coefficient (cm^2/s); FITC, fluorescein-isothiocyanate; Flow FFF, Flow Field-Flow Fractionation; HDL, high density lipoprotein; HSA, human serum albumin; LDL, low density lipoprotein; NBS, N-Benzoyl-staurosporine; t_0, void time (min); t_r, retention time (min); V_0, void volume of the channel (ml); v_0, volumetric crossflow rate (ml/min.) = V(in)-V(out)·V(in), volumetric flowrate at the channel inlet (ml/min.); V(out), volumetric flowrate at the channel outlet (ml/min.); VLDL, very low density lipoprotein; w, channel thickness (μm).

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albumin was from Sigma Chemical Co. (Buchs, Switzerland), α_2-macroglobulin was from Fluka Chemical Co. (Buchs, Switzerland). N-Benzoyl-staurosporine (NBS) was synthesized in house, using patented procedures (United States Patent Nr. 5'093'330). The molecular weight of NBS is 570.65 Da with a log partition coefficient in n-octanol / water of 5.48.

Polysorbate 80® was from ICI Specialty Chemicals (Essen, Germany). Regenerated cellulose 10 kDa membranes from Hoechst, were supplied by Pluess-Stauffer AG (Oftringen, Switzerland). OMEGA, 1 kDa and ALPHA, 3 kDa, both modified polyethersulfone membranes were from Filtron, supplied by Pall AG (Muttenz, Switzerland).

The carrier solution was an isotonic 36 mM phosphate buffer, pH 7.4, with 100 mM sodium chloride as the dominant salt species, containing 0,001% NaN_3 as antimicrobial preservative, with or without 0.005% (w/v) Polysorbate 80®.

Human plasma was prepared from blood from healthy volunteers in fasting state and collected in tubes containing ethylenediaminetetraacetic acid (EDTA). After centrifugation at 3000 g for 30 min. at 6°C, plasma was used fresh or stored at −80°C.

FIELD-FLOW FRACTIONATION

Field-Flow Fractionation is a group of separation methods especially well suited for the separation and characterization of macromolecules and particles. The separation is based on the differential distribution of different sample components across a parabolic flow profile in a thin flat channel and is effected by a force field, applied perpendicularly to the flow profile in the channel (10,12,13). According to the nature of the force field, FFF is subdivided into various types, e.g. Sedimentation, Electrical, Thermal or Flow FFF. Flow FFF is suited for the separation over a wide size range starting with small molecules with a molecular weight of about 5000 Da up to particles of 100 μm in diameter.

In comparison to Symmetrical Flow FFF, where the upper as well as the lower channel walls consist of an ultrafiltration membrane and a frit and the cross flow is externally induced, in Asymmetrical Flow FFF the upper wall of the channel consists simply of a flat float glass plate. The cross flow in Asymmetrical Flow FFF is created by restricting the effluent out of the channel and thus forcing a part of the liquid through the lower wall of the channel, which consists of an ultrafiltration membrane on a porous frit. The separation efficiency is not affected by this modification of the channel, the fluidics setup, however, can be significantly simplified. *Via* the glass plate it is possible to visually detect leaking and separation asymmetry as well as optimizing sample injection and focusing of the sample using colored samples like ferritin.

Samples are transported through the channel with a velocity which is dependent on their average distance to the wall. The distribution of the sample components in the laminar flow is governed by differences in diffusion coefficients. The diffusion coefficients can be calculated from the retention time using equation (1)

$$D = \frac{6t_0 \cdot w^2 \cdot V_c}{t_r \cdot V_0}, \qquad (1)$$

derived from literature (13), where D is the diffusion coefficient, w is the channel thickness, V_c the volumetric crossflow rate (channel inlet flow minus channel outlet flow) and V_0 the void volume of the channel. t_0 is the void time and t_r is the retention time.

Due to the relatively low sample capacity Asymmetrical Flow FFF is very sensitive to sample mass overloading. Sample overloading compromises peak integrity resulting in fronting or tailing of the peaks, zone broadening and altered retention times (15,16). The maximal sample capacity can be determined by monitoring these effects as a function of sample amount.

ASSYMETRICAL FLOW FFF INSTRUMENTATION

The automated system (Fig. 1) mainly consists of two HPLC pumps, a trapezoidal shaped Asymmetrical Flow FFF

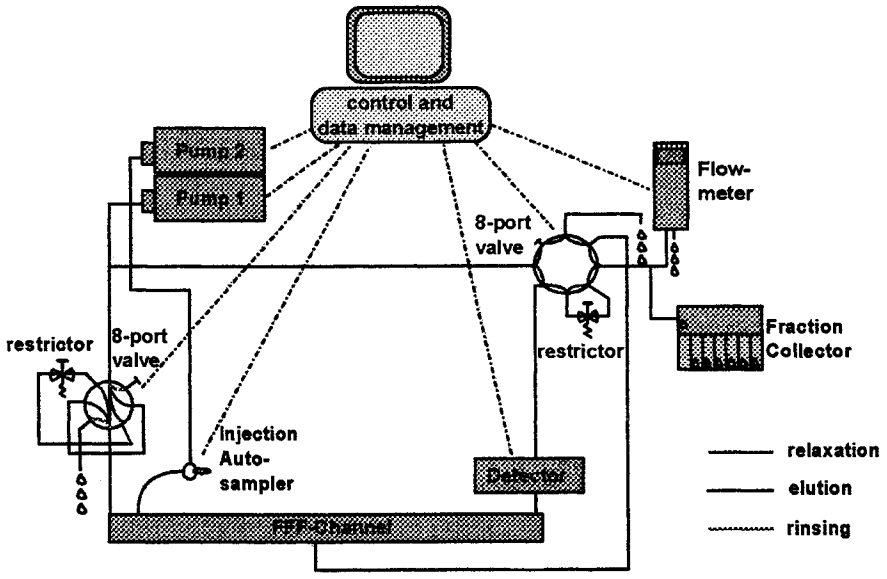

Fig. 1. Schematic Drawing of an Asymmetrical Flow FFF System.

channel, an HPLC compatible UV/VIS detector, a flowmeter (measuring channel outlet flow), two electrically activated valves (to control the directions of flows), manual sample injector or autosampler, and a fraction collector. The system is controlled using adapted HPLC software. The trapezoidal channel was constructed and optimized in house based on a principal design of K.-G. Wahlund, University of Lund (13–16). Channel dimensions are shown in Figure 2. The nominal channel height is 190 μm. Due to swelling of the membrane the real channel height is smaller than the nominal spacer thickness. The actual channel height was determined using test runs, under standard conditions, with purified HSA sample to be 170 μm ± 3 μm (n = 5), using equation (1). The geometrical void volume was 0.46 ml.

Standard operational conditions were: channel inlet flow 4 ml/min., channel outlet flow 1 ml/min., resulting in a crossflow rate of 3 ml/min. All experiments were performed at room temperature (20–22° C). Plasma samples intended for determination of diffusion coefficients were diluted (1:60) before injection, to avoid overloading. The standard Injection volume was 10 microliters. Plasma samples for investigation of drug/plasma protein interactions were not diluted and contained NBS in a concentration range of 2–40 μg/ml. The standard injection volume was 2 microliters containing 4–80 ng of drug.

Specific Properties of N-Benzoyl-staurosporine

Fluorescence

All fluorescence measurements were performed on a Perkin Elmer LS 50 Spectrofluorimeter. Samples were excited at 295 nm where NBS shows a characteristic absorption peak. Quantitation of NBS was calculated from the fluorescence intensity at 378 nm (emission maximum). Excitation and emission spectra are shown in Figure 3.

Apparent Size

Based on its low molecular weight of 570.65 Da, NBS should not be retained by the Flow FFF membranes with cut-offs of 1 kDa to 10 kDa. Due to its strong lipophilic nature, however, NBS can not be dissolved in hydrophilic media; in the injected samples it is always associated with high molecular weight plasma proteins. This apparent increase in molecular weight and size hinders the passage through the membrane. This statement was proved by the analysis of the crossflow, where no NBS could be detected.

RESULTS

In order to study drug plasma protein interactions using Asymmetrical Flow FFF, first the separation characteristics of this technique had to be investigated. In that respect, the yield of the drug in the collected fractions and the degree of adsorption of sample components on the membrane are of importance to know.

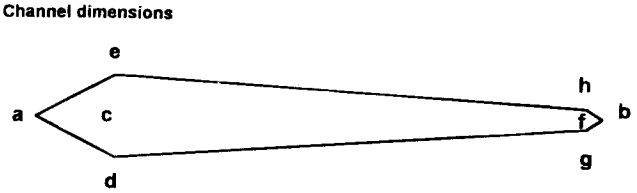

Fig. 2. c = sample inlet, b = sample outlet, a–b = 281 mm, a–c = 15 mm, d–e = 15 mm b–f = 5 mm, g–h = 5 mm.

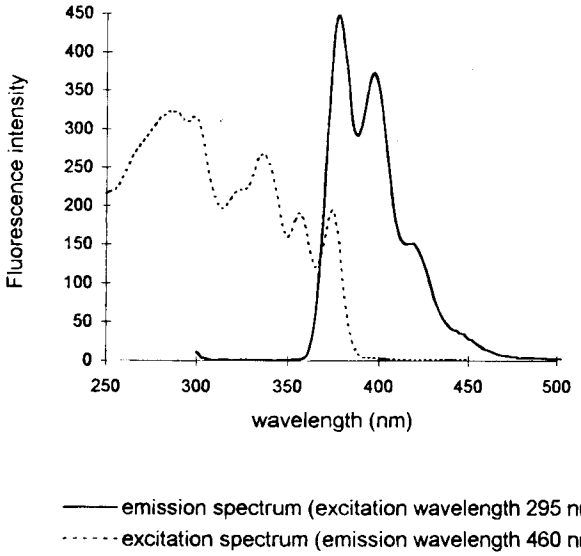

Fig. 3. N-Benzoyl-Staurosporine, excitation and emission spectra.

Influence of Membrane Type and Carrier Solution Composition on Recovery

It is known that highly diluted protein samples may suffer from a considerable loss of protein due to sample adsorption at surfaces of equipment (18). Since the FFF process results in a sample dilution in the order of 1000, control and limitation of adsorption effects are of high importance.

Some experimental factors important for Asymmetrical Flow FFF separations such as membrane type, carrier solution, and temperature were already investigated by Wahlund using model proteins (ferritin and HSA) and a virus (15). These investigations were performed to determine diffusion coefficients, retention behavior and peak shape. Recoveries, however, were not investigated. Therefore studies on the influence of membrane type and carrier liquid composition on recovery of drug and proteins had to be performed before drug/plasma protein interactions were analyzed. The importance of knowing and controlling the adsorption phenomena of the different compounds became obvious in an experiment where N-Benzoyl-staurosporine was eluted in a Asymmetrical Flow FFF system using a regenerated cellulose membrane and phosphate buffer as carrier liquid. No NBS could be found in any of the FFF fractions but still 70% of FITC-albumin (in a second experiment using the same conditions) could be recovered in the outlet flow. Obviously, in this case, no interpretation on the degree of drug protein interaction is possible. To prove that NBS was lost due to adsorption on the membrane, the membrane was cut into pieces of 1 centimeter and individually extracted. The extracts were analyzed by fluorescence for presence of NBS (Fig. 4). The data indicated that NBS was totally adsorbed on the membrane. The largest amount of the drug was found close downstream to the injection spot, where the sample is focused during the injection.

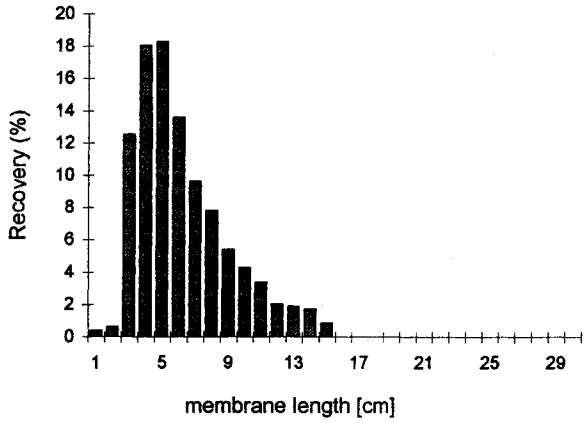

Fig. 4. Adsorption of NBS on the regenerated cellulose membrane. Experimental conditions of the Flow-FFF procedure: carrier liquid: 36 mM phosphate buffer pH 7.4, containing 100 mM sodium chloride; flow rates: V (in) = 4 ml/min., V (out) = 1 ml/min; injection volume: 10 µl; for further experimental details see methods section.

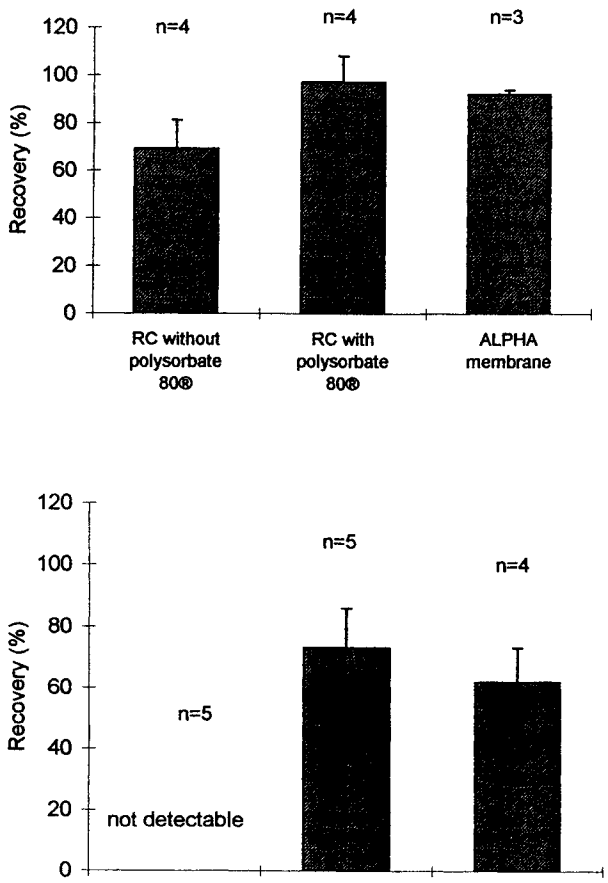

Fig. 5. Recoveries of FITC-albumin (top) and NBS (bottom). Membrane types: RC (reg. cellulose 10 kDa cutoff) and ALPHA membrane 3 kDa cutoff. The carrier liquid was 36 mM phosphate buffer pH 7.4, containing 100 mM sodium chloride, and Polysorbate 80® in phosphate buffer 0.005% (m/m); flow rates: V (in) = 4 ml/min., V (out) = 1 ml/min; injection volumes: 10 and 20 µl.

Therefore, we investigated the recovery of the lipophilic drug NBS as well as FITC-albumin as a fluorescently labeled model plasma protein on different membranes, and using two carrier liquid compositions. The strong adsorption losses of NBS on the ultrafiltration membrane could not be reduced by using a so called low protein binding OMEGA membrane (1 kDa cut-off). NBS could be detected neither in the eluted fractions nor in the crossflow liquid. However, with the ALPHA membrane (3 or 10 kDa cut-off, respectively), adsorption of NBS was significantly reduced. The same effect was observed using a regenerated cellulose membrane and adding 0.005% (w/v) Polysorbate 80® to the carrier solution (see Fig. 5).

From these experiments it can be concluded that for proteins like HSA the typical loss of material in the range of 30% on regenerated cellulose membranes can be significantly reduced down to about 3% by addition of 0.005% (m/V) Polysorbate 80® to the carrier liquid. Alternatively the adsorption could also be reduced down to about 8% by the use of a hydrophilic membrane like that of the ALPHA type. In this case no additional detergent is needed.

In the case of NBS the interactions with the regenerated cellulose membrane are much stronger, as no NBS can be found in any of the fractions. Here the addition of Polysorbate 80® or the use of the ALPHA membrane with more hydrophilic characteristics dramatically reduces the adsorption and about 70% of drug can be detected in the fractions (Figure 5). To avoid potential changes in lipoprotein structure due to interactions with the detergent, the ALPHA membrane and phosphate buffer without Polysorbate 80® as carrier liquid were selected for the further experiments.

Separation of Human Plasma in Different Protein Fractions

In an optimized Asymmetrical Flow FFF system, using an ALPHA membrane and isotonic phosphate buffer as carrier solution, protein standards were injected for calibration purposes (Fig. 6). In Fig. 7 a typical fractogram of fresh human plasma on an optimized system is shown. Four different peaks can be discriminated. From the peak positions, diffusion coefficients of both the protein standards and the different fractions of fresh human plasma were calculated using equation 1 and compared with literature values (Table I).

Data from Fig. 6 were correlated to those from Fig. 7 assuming identical retention behavior under same separation conditions. After injection of diluted, but not further treated plasma and separation in the Asymmetrical Flow FFF system, HSA, HDL, α_2-macroglobulin and LDL could be fractionated. VLDL was not detectable under the conditions used. The fractograms of α_2-macroglobulin and LDL standards additionally show a HSA peak, indicating that these protein standards were contaminated with small amounts of HSA.

Investigation of Drug/Protein Interactions

For this experiment the optimized conditions as described above (ALPHA membrane, carrier solution without Polysorbate 80®) were used. The fractions were analyzed with respect to their NBS concentration by fluorescence spectroscopy. Figure 8 shows a typical fractogram of human plasma containing NBS. In the overlay the yield of NBS in the fractions collected is shown. These data clearly show that NBS binds only to the HSA fraction (62% of the injected amount).

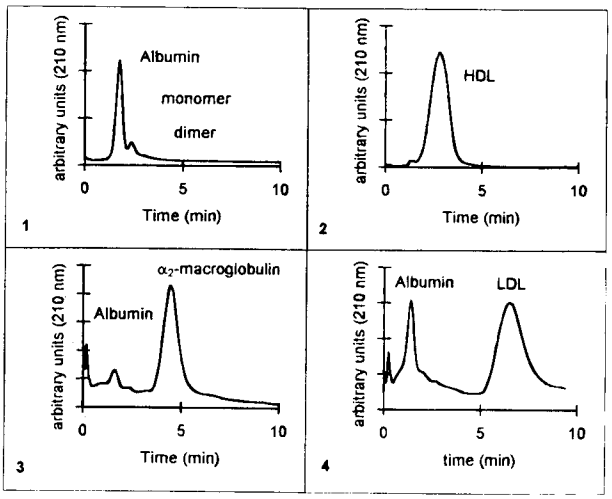

Fig. 6. Fractionation of plasma protein standards. 1, HSA; 2, human HDL (separated by Ultracentrifugation, density range 1.063–1.2g/ml); 3, human α_2-Macroglobulin; 4, human LDL (separated by Ultracentrifugation, density range 1.02–1.063g/ml). Experimental conditions of the Flow-FFF procedure: carrier liquid: 36 mM phosphate buffer pH 7.4, containing 100 mM sodium chloride. Membrane: ALPHA membrane 3 kDa; flow rates: V (in) = 4 ml/min., V (out) = 1 ml/min.; injection volume: 10 µl; for further experimental details see methods section.

Table I. Diffusion Coefficients of Protein Standards and Plasma Components, Experimentally Determined and Calculated from Equation 1, and Compared to Literature Values (18,19)

Protein fraction	Diffusion coefficients (20 °C) of human plasma proteins/10^{-7} $cm^2 \cdot s^{-1}$	
	experimentally determined values[a]	literature values[c]
HSA (Sigma)	6.1[b] +/− 0.4	6.1 (19)
Plasma peak 1	6.4 +/− 0.3	
HDL (Calbiochem, density from 1.2–1.063 g/ml)	4.0 +/− 0.3	3.9 HDL_3(20) 3.7 HDL_2(20)
Plasma peak 2	4.1 +/− 0.2	
α2 Macroglobulin (Sigma)	2.2 +/− 0.1	2.4 (19)
Plasma peak 3	2.3 +/− 0.1	
LDL (Calbiochem, density from 1.063–1.02 g/ml)	1.7 +/− 0.04	1.6–1.8 (20)[d]
Plasma peak 4	1.7 +/− 0.06	

Note: Experimental conditions of the Flow-FFF procedure: carrier liquid: 36 mM phosphate buffer pH 7.4, containing 100 mM sodium chloride; membrane: ALPHA membrane 3 kDa; flow rates: V (in) = 4 ml/min., V (out) = 1 ml/min; injection volume: 10 µl; for further experimental details see methods section.
[a] Average values +/− S.D.
[b] Used for calculation of channel height.
[c] Determined by analytical ultracentrifugation studies.
[d] Pig LDL.

DISCUSSION

Influence of Membrane Type and Carrier Solution Composition on Recovery

Prerequisite for a reliable Asymmetrical Flow FFF separation is basic knowledge of adsorption of the sample components on the FFF membrane and recovery of the fractions. It could be demonstrated that for HSA (hydrophilic protein) and NBS (lipophilic drug) not only membrane type but also carrier solution composition can strongly influence the amount of adsorption of the samples.

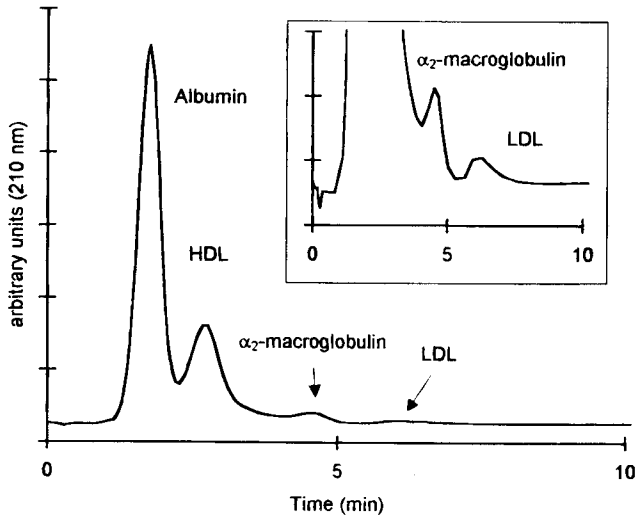

Fig. 7. Fractogram of fresh human plasma. Fresh human plasma, diluted (1:60) with 36 mM phosphate buffer pH 7.4, containing 100 mM sodium chloride was injected without further sample preparation. Injection volume: 10 µl. Experimental conditions of the Flow-FFF procedure: carrier liquid: 36 mM phosphate buffer pH 7.4, containing 100 mM sodium chloride. Membrane: ALPHA membrane 3 kDa. Flow rates: V (in) = 4 ml/min., V (out) = 1 ml/min. For further experimental details see methods section.

FITC-albumin had an acceptable recovery of 70% using a membrane consisting of regenerated cellulose. However, by addition of Polysorbate 80® to the carrier solution, the recovery could be increased to close to 100% (Fig 5). HSA concentration during Asymmetrical Flow FFF separation is in the range of micrograms per milliliter. NBS in contrast showed strong adsorption to the cellulose membrane. No substance could be detected in any of the eluted fractions. After addition of Polysorbate 80® to the carrier solution adsorption was significantly reduced from 100% to about 27%. However, NBS concentration during Asymmetrical Flow FFF separation is in the range of nanogram per milliliter, which is a factor thousand lower than HSA concentration. The generally higher tendency of NBS to be adsorbed on both membranes investigated here is most likely due to its lipophilic nature as well as to the low concentration used in the experiments. As for the latter, it is a well known fact that loss due to adsorption increases with decrease in concentration (17). Polysorbate 80® is a non ionogenic surfactant, used to prevent adsorption by suppressing hydrophobic interactions between sample and membrane. Although the use of Polysorbate 80® can successfully reduce the adsorption, it should be kept in mind that due to its surface active properties it may change tertiary protein structure and/or influence stability of lipoprotein/drug complex during fractionation. The ALPHA

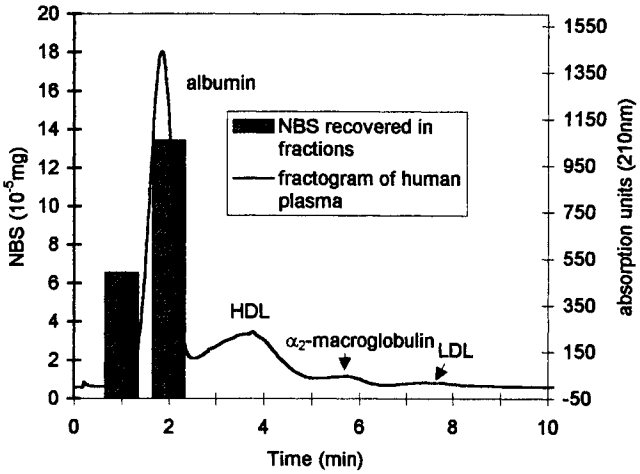

Fig. 8. Interaction of NBS with plasma proteins. 2 µl of human plasma containing 50 ng of NBS (sample concentration 25 µg/ml) were injected in the Asymmetrical Flow FFF system undiluted and without further sample preparation. Fractions were collected every minute. Experimental conditions of the Flow-FFF procedure: carrier liquid: 36 mM phosphate buffer pH 7.4, containing 100 mM sodium chloride; Flow rates: V (in) = 4 ml/min., V (out) = 1 ml/min., for further experimental details see methods section.

membrane proved to be suitable to suppress the adsorption for both lipophilic drug and hydrophilic protein without the necessity to add Polysorbate 80® to the carrier liquid (Figure 8) and was therefore judged to be optimal for this kind of experiments.

Separation of Human Plasma in Different Protein Fractions

According to the underlying theoretical separation principle of Flow FFF, the diffusion characteristics of the sample components determine their retention times. Calculation of diffusion coefficients using retention time and crossflow enables the comparison of experimental values with literature values. In Table I, experimental diffusion coefficients of protein standards and human plasma components and the corresponding literature values are listed. The data indicate that the peaks of human plasma correspond to the following protein fractions:

Plasma peak 1 contains mainly HSA. Plasma peak 2 consists of HDL_3 and HDL_2 subfractions. Plasma peak 3 is α_2-macroglobulin. Plasma peak 4 contains the LDL fraction. Not only the experimental values of diffusion coefficients of the protein standards obtained after Asymmetrical Flow FFF analysis but also the experimental diffusion coefficients of the peaks of plasma obtained after Asymmetrical Flow FFF analysis correspond well with literature values.

As particles are separated in Asymmetrical Flow FFF on the basis of their diffusion coefficients, it is not possible to differentiate between two different proteins with very similar diffusion coefficients. For a proper interpretation of the fractogram it has therefore to be kept in mind that the "HSA fraction" not only contains HSA, but because the sample was injected without any preparation, may contain additionally small amounts of other plasma proteins with similar diffusion coefficients and molecular weights. For instance α_1-acidic glycoprotein (MW 40'000, D = $5.3 \cdot 10^{-7}$ $cm^2 \cdot s^{-1}$ (19)) and α_1-antitrypsin (MW 54'000, D = $5.2 \cdot 10^{-7}$ $cm^2 \cdot s^{-1}$ (19)) are present in a concentration of 1–4%, and 4–12%, respectively, relative to HSA. In this example resolution is additionally complicated by the differences in concentration. As a consequence, these proteins are fractionated together with HSA. It is therefore not possible to distinguish whether a drug, found in this fraction shows affinity solely to HSA and/or to an other plasma protein present in this fraction.

Investigation of Drug/Protein Interactions

A typical fractogram of NBS in human plasma is shown in Figure 8.

All bound NBS is detected in the HSA fraction.

As mentioned in the Methods section, Asymmetrical Flow FFF is very sensitive to sample mass overloading leading to changes in peak shape and retention time. The maximum amount of sample the system can tolerate was evaluated by injecting a sample in different concentrations. However, to be able to detect NBS in the fractions, a sufficient amount of sample has to be injected. In our work, the NBS containing plasma sample intended for investigation of drug/protein interactions, was not diluted before injection. As a consequence, some overloading effects are perceivable in Figure 8. The peaks are broadened and retention times of plasma proteins are higher compared to Figure 6. However, the protein fractions are still distinguishable and NBS can be detected in the fractions without the necessity of concentration steps. For experiments intended for measurement of diffusions coefficients, the protein samples were diluted in order to avoid overloading.

The results shown in Figure 8 illustrate the suitability of Asymmetrical Flow FFF to study affinity of drugs to plasma proteins (provided that Asymmetrical Flow FFF is performed under optimal conditions). The spontaneous drug distribution within the natural occurring environment after intravenous administrations and with minimal perturbation of the equilibrium existing between plasma carriers and drugs can be studied without sample preparation.

Comparison of Asymmetrical Flow FFF with Other Methods Concerning Plasma Protein Separation

In order to assess the usefulness of Asymmetrical Flow FFF, alternative separation techniques have to be compared. Analytical density gradient ultracentrifugation is an established method to separate plasma lipoproteins. In contrast to Asymmetrical Flow FFF it enables preparative separation of lipoproteins, but the samples are exposed to high salt concentrations for hours and thus have to be further treated before use. Asymmetrical Flow FFF separates plasma proteins much faster than ultracentrifugation and under more physiological conditions. A major advantage of Asymmetrical Flow FFF, compared to ultracentrifugation, is that exchange of lipid and apolipoprotein between lipoprotein classes, which is known to happen during the ultracentrifugation procedure (5), is rather unlikely to happen due to the short separation time.

Further methods to separate plasma proteins and lipoproteins and to study their interactions with drugs are adsorption chromatography, size exclusion chromatography and electrophoresis. Adsorption chromatography alone is not capable to

separate the main lipoprotein classes while size exclusion chromatography requires a preceding ultracentrifugation step (6,7). Quantitation of drug after agarose gel electrophoresis in the separated protein fractions is time consuming and arduous.

CONCLUSION

Asymmetrical Flow FFF is a fast and convenient method to characterize drug/plasma protein interactions, as shown at the N-Benzoyl-staurosporine model, if experimental conditions are thoroughly selected and controlled to suppress drug and plasma protein adsorption at the ultrafiltration membrane of the apparatus

Asymmetrical Flow FFF is able to fractionate human plasma into four major protein classes: HSA, HDL, α_2-macroglobulin and LDL with high efficiency in less than 10 minutes. No sample preparation is required; experimental diffusion coefficients of plasma fractions and protein standards were nearly identical to literature values.

Asymmetrical Flow FFF allows the study of drug/protein interactions under near native conditions without sample preparation and thus reflects more likely the actual *in vivo* situation. For analytical purposes it has the potential to rival established technologies such as ultracentrifugation in terms of ease-of-use, precision, and short analysis time.

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